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1 **Distribution of pines in the Iberian Peninsula agrees with species**
2 **differences in foliage frost tolerance, not with vulnerability to freezing-**
3 **induced xylem embolism**

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6 **Laura Fernández-Pérez^{1*}, Pedro Villar-Salvador¹, Jordi Martínez-Vilalta^{2,3}, Andrei**
7 **Toca¹, Miguel A. Zavala¹**

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12 ¹Forest Ecology and Restoration Group. Departamento de Ciencias de la Vida, Universidad de
13 Alcalá, Apdo. 20, Alcalá de Henares, 28805 Madrid, Spain

14 ² CREAM, Cerdanyola del Vallès, Barcelona, Spain

15 ³Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain

16
17 *Author of contact: laura.fernandezp@edu.uah.es/ +34 918 85 6407

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19 **Running head:** Frost physiology of European pines

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24 **Abstract**

25 Drought and frosts are major determinants of plant functioning and distribution. Both stresses
26 can cause xylem embolism and foliage damage. The objective of this study was to analyze if
27 the distribution of six common pine species along latitudinal and altitudinal gradients in
28 Europe is related to their interspecific differences in frost tolerance and to the physiological
29 mechanisms underlying species-specific frost tolerance. We also evaluate if frost tolerance
30 depends on plant water status. We studied survival to a range of freezing temperatures in two-
31 year-old plants and assessed the percentage loss of hydraulic conductivity (PLC) due xylem
32 embolism formation and foliage damage determined by needle electrolyte leakage (EL) after a
33 single frost cycle to -15 °C and over a range of predawn water potential (ψ_{pd}) values. Species
34 experiencing cold winters in their range (*P. nigra*, *P. sylvestris* and *P. uncinata*) had the
35 highest frost survival rates and lowest needle EL and soluble sugar concentration (SS). In
36 contrast, the pines inhabiting mild or cool winter locations (especially *P. halepensis* and *P.*
37 *pinea* and, to a lower extent, *P. pinaster*) had the lowest frost survival and highest needle EL
38 and SS values. Freezing-induced PLC was very low and differences among species were not
39 related to frost damage. Reduction in ψ_{pd} decreased leaf frost damage in *P. pinea* and *P.*
40 *sylvestris*, increased it in *P. uncinata* and had a neutral effect on the rest of the species. This
41 study demonstrates that freezing temperatures are a major environmental driver for pine
42 distribution and suggests that interspecific differences in leaf frost sensitivity rather than
43 vulnerability to freezing-induced embolism or SS explain pine juvenile frost survival.

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49 **Introduction**

50 Drought and frosts are major drivers of plant evolution and distribution (Woodward and
51 Williams 1987, Pockman and Sperry 1997, Choat et al. 2012). Both stress factors affect
52 important plant physiological processes (Sakai and Larcher 1987, Mayr et al. 2006).
53 Particularly, water transport and gas exchange are usually reduced due to frost-induced
54 embolism and drought-induced stomatal closure (Sperry and Sullivan 1992, Davis et al. 1999,
55 Willson and Jackson 2006). These physiological responses reduce the productive capacity and
56 hydration of plants, might damage the plant, and eventually cause their death (McCulloh et al.
57 2011, McDowell 2011, Peguero-Pina et al. 2011). Most comparative studies on frost tolerance
58 use foliage electrolyte leakage or decrease in fluorescence activity of photosystems to assess
59 tissue damage (Strand and Öquist 1988, Climent et al. 2009), while few studies report plant
60 survival (Hawkins et al. 2003), which is key for plant fitness. However, while simple and fast,
61 electrolyte leakage or fluorescence of photosystems do not inform on the capacity of
62 individuals to remain alive after a stress event.

63 The mechanism by which drought and frosts cause xylem embolism is different
64 (Zimmermann, 1983), but in both cases the amount of embolism is expected to increase with
65 declining xylem water potential (ψ) (Davis et al. 1999, Mayr et al. 2003). Drought-induced
66 embolism occurs when low ψ causes the air contained in air filled cells to be sucked through
67 the pits of water-filled xylem conduits, forming bubbles that block the conduit (Zimmermann,
68 1983). Frost-induced embolism occurs when the bubbles formed after thawing of frozen water
69 inside the xylem conduits expand and block xylem conduits (Robson *et al.*, 1988; Langan *et*
70 *al.*, 1997).

71 Frosts can also damage plants by causing the loss of cell membrane functional
72 integrity (Uemura et al. 2006), which mainly occurs in the most exposed organs. Freezing of
73 apoplast causes the water to move outside the cell producing, large changes in protoplasm

74 volume, which can damage the cell membrane and hinder metabolism (Wesley-Smith et al.
75 2015, Sakai and Larcher 1987). Soluble sugars (SS) play an important role in plant frost
76 tolerance. SS increase the stability of cell membranes to frost damage (Uemura and Steponkus
77 2003), may contribute to embolism repair (Zwieniecki and Hollbrook, 2009) and reduce the
78 protoplasm osmotic potential, which increases cell desiccation tolerance and reduces the
79 freezing point (Hinesley et al. 1992). Finally, plants use stored SS to support winter
80 metabolism and early spring growth (Uscola et al. 2015). Consequently, many plants from the
81 cold and temperate biomes increase their SS concentration during frost acclimation in the fall
82 to a maximum level in midwinter, which is then reduced in spring and summer with cold
83 deacclimation (Sakai and Larcher 1987, Martínez-Vilalta et al. 2016).

84 Distribution of pine species native to Europe appears segregated along latitudinal and
85 altitudinal gradients in a predictable manner suggesting the existence of environmental factors
86 that differentially influence the species. Some species, namely *P. halepensis* Mill., *P. pinaster*
87 Ait., and *P. pinea* L., are concentrated in southern of Europe, particularly at Mediterranean
88 climate sites with mild to cool winters and hot and dry summers (Figure S1). Other species,
89 such as *Pinus nigra* J.F. Arnold, *Pinus sylvestris* L. and *Pinus uncinata* Raymond ex A. DC.,
90 are located in cold winter sites, either at high latitude (in central and northern Europe) or at
91 high altitude (southern Europe including the Iberian Peninsula, Figure S1) (Richardson,
92 1998). In these high mountain locations rainfall can be higher, evapotranspiration is lower and
93 thus summer drought is shorter and of moderate intensity relative to lower altitude sites. Intra-
94 and inter-specific variability to drought-induced embolism is low in pines (Lamy et al. 2014;
95 Martínez-Vilalta and Piñol 2002; Martínez-Vilalta et al. 2004, Martínez-Vilalta et al. 2009),
96 which make differences in drought-induced embolism an unlikely explanation for differences
97 in species distribution. We hypothesize that this latitudinal and altitudinal distribution pattern
98 of European pines could be driven by species differences in cold tolerance.

99 The Mediterranean climate has a dry season concentrated in the summer but
100 occasionally winters can also be dry. High-altitude locations in the Mediterranean basin such
101 as the plateaus and mountain ranges of the Iberian Peninsula have cold winters. The
102 combination of cold temperatures in winter and summer drought is an important determinant
103 for plant life in these environments (Mitrakos, 1980). Besides, in dry winters years, plants at
104 Mediterranean cold-winter sites can suffer drought stress (Peguero-Pina et al. 2011), which
105 can promote the negative effects of frost-induced embolism (Willson and Jackson, 2006).
106 This phenomenon has been related to pine die-back in high elevations in the Mediterranean
107 mountains and boreal forests (Kullman 1991, Peguero-Pina et al. 2011). At the same time,
108 drought stress can also increase frost tolerance in some plant organs (Medeiros and Pockman
109 2011, Villar-Salvador et al. 2013, Sperling et al. 2017). It is possible that drought-induced
110 physiological acclimation responses such as changes in SS concentration, cell membrane
111 stability or the reduction of cell osmotic potential (Serrano et al. 2005, Beck et al. 2007,
112 Villar-Salvador et al. 2013) may also enhance cold tolerance. Numerous studies have
113 addressed the effect of drought and frost independently on plant physiological performance
114 (see Beck et al. 2004, Dobbertin et al. 2005), but relatively few studies have analyzed
115 experimentally how drought affects frost tolerance of plants (Grossnickle et al. 1991,
116 Medeiros and Pockman 2011).

117 The general objective of this study is to assess whether interspecific differences in
118 frost tolerance are related to the distribution of the six-pine species native to the Iberian
119 Peninsula and the physiological mechanisms underlying species differences in frost tolerance.
120 These pine species (*P. halepensis*, *P. pinea*, *P. pinaster*, *P. nigra*, *P. sylvestris* and *P.*
121 *uncinata*) are also common throughout Europe. Specifically, we asked: 1) Do pine species
122 inhabiting cold sites have higher inherent cold tolerance than pines inhabiting mild or cool
123 winter locations? 2) Are differences in frost tolerance explained by differences in frost-

124 induced embolism, needle frost tolerance or tissue SS? 3) What is the effect of drought stress
125 on frost tolerance? To answer these questions, we conducted an experiment in which the frost
126 survival, and the interaction between drought and frost on xylem embolism and needle
127 damage was assessed in two-year-old plants. We used juveniles as early life stages are
128 important bottlenecks in pine population dynamics (Castro 1999, Herrero et al. 2013).

129

130 **Methods**

131 **1. Plant material.**

132 Seeds of pine species were collected in populations of the Iberian Range (Eastern Spain),
133 except for *P. uncinata*, whose seeds were collected in the Pyrenees (Table 1). Seeding was
134 done in 2012 on different dates to synchronize seedling emergence. *Pinus halepensis* was
135 seeded on January 30th, while *P. pinaster* and *P. pinea* were seeded 7 and 11 days later,
136 respectively than *P. halepensis*. *Pinus nigra* subsp. *salzmannii* (here after *P. nigra*) and *P.*
137 *uncinata* were seeded on February 15th while *P. sylvestris* was seeded on February 20th.
138 Seedlings were cultivated in Plasnor trays (190/300-45, Plasnor, Spain) with 45 cavities of
139 300 ml. Growing medium was fertilized peat (White 420 F6 Kekkilä, Finland; pH 4.7,
140 containing 0.8-1 kg/m³ of a slow release fertilizer NPK 16-10-20). Seedlings were initially
141 grown in a greenhouse of the Centro Nacional de Recursos Genéticos Forestales “El
142 Serranillo” (Guadalajara, Spain) to avoid frost damage. In mid May 2012, seedlings were
143 moved outdoors and were kept well-watered, periodically fertilized and exposed to the natural
144 seasonal changes in temperature, radiation and day length until January 2014.

145

146 **2. Testing differences in seedling frost survival**

147 Frost tolerance in the experiment was assessed by seedling survival, xylem embolism and
148 needle damage. Survival was assessed after freezing batches of seedlings at the following

149 target temperatures: -5, -8, -12, -16, -20, -25 and -30 °C. For each temperature, we used 10
150 seedlings per species, which were transplanted into styrofoam containers to isolate the roots.
151 Plants were placed in a programmable freezing chamber (Dycometal, CCK, Spain) at the
152 INIA (Madrid, Spain), and subjected to one frost cycle. Temperature was reduced from 5 °C
153 to 0 °C in 1 h. Then, the temperature decreased at a ~ 3 °C h⁻¹ rate to the target temperature,
154 which was maintained for 3 h. Finally, temperature was increased at a rate of 5 °C h⁻¹ up to 5
155 °C, where temperature was maintained for 1 h. Then, seedlings were moved to a greenhouse
156 and were well-watered to favor their recovery. After 2 months, we verified seedling status and
157 we considered a seedling to be dead when it had not resumed growth and >95% of needles
158 where brown.

159

160 **3. Foliage electrolyte leakage and xylem embolism measurements**

161 In early March 2014, we performed an experiment in which seedlings of all species were
162 randomly distributed into four treatments: 1) Frost and well irrigated, 2) Frost with restricted
163 watering, 3) Unfrozen and well irrigated and 4) Unfrozen with restricted watering. We used
164 14-18 seedlings per species and treatment. The sizes of shoot fractions of the seedlings used
165 in the experiment are presented in Table 2. Restricted watering was achieved by stopping
166 watering 7-10 days before physiological measurements. The remaining seedlings were
167 irrigated every 2-3 days to keep them well-watered. For the frost treatments, seedlings were
168 subjected to a single -15 °C frost cycle using a programmable freezing chamber (A.S.L.
169 Aralab International®-CON-550-20, Madrid, Spain), using the same protocol explained in the
170 previous section. Before frost exposure, we measured seedling predawn water potential (ψ_{pd})
171 using a Scholander Pressure Chamber (SKPM 1400, Skye Instruments, Llandrindod Wells,
172 UK). In most cases, the ψ_{pd} was measured on lateral twigs but in a few cases, we used needles
173 attached to brachiblasts. Seedlings were covered with opaque plastic bags overnight to reduce

174 seedling transpiration before ψ_{pd} measurements. ψ_{pd} ranged between -0.23 to -1.68 MPa in *P.*
175 *nigra*, *P. sylvestris* and *P. uncinata*, and -0.16 to -2.0 MPa in *P. halepensis*, *P. pinaster* and *P.*
176 *pinea*.

177 We measured damage in secondary needles and xylem embolism after the -15 °C frost
178 to assess the importance of the interaction of frost and drought on species physiological
179 performance. Needle frost damage was assessed by electrolyte leakage (EL) (Earnshaw
180 1993), while xylem frost-induced embolism was measured as the percentage loss in stem
181 hydraulic conductivity (PLC) (Charrier et al. 2013). For needle EL measurements, secondary
182 needles were cut in small pieces (fresh weight ~0.30 g) after the -15 °C treatment, and washed
183 twice in distilled water for 20 min and left in a vial with 20 ml of deionized water that was
184 gently shaken (Orbital shaker, Selecta, Spain) on an illuminated bench under laboratory
185 temperature (20-22 °C). After 24 h, the electrical conductivity (C_i) of the water bathing the
186 needles was measured with an electro conductivity meter (Crison® CM 35+, Spain). Then,
187 the needles were autoclaved at 120 kg cm² for 10 min. After cooling the samples at room
188 temperature, electrical conductivity was remeasured (C_f). EL was calculated as:

189 EL=

190 $\left(\frac{C_i}{C_f} \times 100\right)$(Equation 1).

191 PLC was measured in stem segments 3 cm long excised from the lower part of the
192 stem (immediately above the cotyledon insertion point). Working under water to prevent air
193 entry into the tracheids, we cut the stem segments, removed the bark in the 3-4 mm extremes
194 of the segments and then recut the stems to remove resin remains with a fresh razor blade
195 under water. Water flow through the stem segments was measured using the Xyl'em Plus
196 apparatus (Embolism Meter, INRA Licensed Instrumentec, France, Version 2.1, Cochard,
197 2002). Previously, the extremes of the stems segments were wrapped with Teflon tape before
198 connection to the tubes to prevent lateral leaks. We calculated the PLC as:

199 $PLC = \frac{K_m - K_i}{K_m} \times 100$ (Equation 2),

200 where K_i ($\text{mmol m s}^{-1} \text{MPa}^{-1}$) is the hydraulic conductivity of the segment after seedling
201 freezing and before embolism removal and K_m was the maximum hydraulic conductivity,
202 measured after removing embolism by immersing the stem segments in the same solution
203 used for the flow measurements in a vacuum chamber (BR116, Selecta, Barcelona, Spain) for
204 at least 12 h at a suction of 90 kPa. Both conductivities were calculated as the ratio between
205 the flow of a 10 mmol KCl + 1 mmol of CaCl₂ solution through the segment divided by the
206 pressure gradient (pressure difference = 0.49 - 0.52 kPa). In all cases, the solution used for
207 measurements was previously filtered with a 0.45 μm nylon syringe filter membrane (Filter-
208 Lab, Barcelona, Spain) and degassed in the mentioned vacuum chamber for 24 h at a suction
209 of 90 kPa.

210

211 **4. Measurement of tracheid diameter**

212 Tracheid diameter was measured only in the unfrozen, well-watered plants. Cross-sections 20
213 μm in thickness were cut from six individuals per species using a cryostat microtome
214 (Microm HM 505 E, Ramsey, MN, USA). We used the same stem segments used for PLC.
215 Cross-sections were bathed in bleach for 30 min, rinsed in tap water and finally stained with
216 safranin (1 g in 50% ethanol). Tracheid diameter was measured using the ImageJ software on
217 all the tracheids located in three randomly selected radial rows, from the pit to the outer
218 border of the section. A minimum of 160 tracheids per individual was measured.

219

220 **5. Soluble sugars determination.**

221 Soluble sugars (SS) were determined only in unfrozen well-watered plants. Needles of six
222 seedlings per species were stored frozen immediately after EL measurements. When SS were
223 determined, needles were thawed and dried at 50 °C for 24 h. SS were extracted following the

224 methodology in Chow and Landh usser (2004). Briefly, needles were ground in a ball mill
225 (PM100, Retsch, Haan, Germany) and 50 mg of ground needles was used for SS extraction
226 with 5 ml of ethanol 80% at 90  C for 10 min. Then, the tubes were centrifuged at 2500 rpm
227 for 5 min, and the supernatant was preserved. We repeated the extraction three times and the
228 supernatant of the three extractions was combined and oven-dried at 50  C until complete
229 evaporation of the ethanol. Then, the residue was dissolved in 1 ml hot deionized water and
230 filtered with 0.45  m nylon syringe filter membrane (Filter-Lab, Barcelona, Spain).

231 We analyzed the concentration of main SS (see Table 2) using a High Performance
232 Liquid Chromatography system (Agilent Technologies, 1100 series, Palo Alto, CA, USA)
233 coupled to the refractive index detector (HPLC-RI) equipped with a quaternary pump,
234 degasser, auto injector and HP-1047A RI detector. The chromatographic data were acquired
235 using the ChemStation software. The samples were analyzed using a Supelcogel column Ca-
236 59305U (30 cm x 7.8 mm) under isocratic condition with ultrapure water (Milli-Q). The
237 injection volume was 20  L and the flow rate was 0.5 mL min⁻¹. Column and detector
238 temperature were 80 and 50  C, respectively. Identification and quantification of sugars was
239 determined by comparing the peak retention time and area of the samples with pure standard
240 solutions of each sugar that were made by us in the laboratory.

241

242 **6. Data analysis**

243 To compare the frost survival of pine species, we compared the temperature at which survival
244 is 50% (LT₅₀). We used a logistic model to fit survival data for each species against freezing
245 temperatures (see Figure 1). LT₅₀ is the inflection point of the logistic model. We used a
246 logistic model to fit survival data for each species against freezing temperatures (see Figure
247 1). For each LT₅₀ value, we calculated its 95% confidence intervals following Collet (1991).

248 A generalized linear model (GLM) was performed to test the effect of species (six
249 levels), frost (two levels, unfrozen and -15 °C frost) and ψ_{pd} on EL and PLC, testing all the
250 interactions. As the Species \times Frost \times ψ_{pd} was significant for EL, the least square means and
251 standard errors were calculated at specific ψ_{pd} values, -0.4, -1.2 and -1.5 MPa (see Figure 2).

252 A general linear mixed model (GLMM) was performed to compare the tracheid
253 diameter among species. Data of tracheids in a row were nested within the individual, which
254 was included as a random effect. Species differences in SS were analyzed using one-way
255 ANOVA. Tukey's Honest Significance Difference (HSD) test ($\alpha=0.05$) was used for multiple
256 comparisons of mean values. All data was checked for residual normal distribution (Shapiro-
257 Test) and variance homoscedasticity (Levene's test. Statistical analyses were performed in R
258 platform and Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA).

259

260 **Results**

261 *Survival*

262 In all species, fitted curves of survival against freezing temperatures were highly significant
263 ($P<0.001$). Frost survival significantly differed among species (Figure 1) with a two-fold LT_{50}
264 variation between *P. halepensis*, the species with the lowest LT_{50} , and *P. nigra*, the species
265 with the highest LT_{50} . *Pinus nigra*, *P. sylvestris* and *P. uncinata* did not show any mortality
266 until -15 °C, while at this temperature survival of *P. halepensis* and *P. pinea* was only around
267 20%. Four groups according to their LT_{50} values and confidence intervals were distinguished:
268 *P. halepensis*=*P. pinea*>*P. pinaster*>*P. sylvestris*=*P. uncinata*>*P. nigra*. The survival curve
269 was similar for *Pinus sylvestris* and *P. uncinata*, and their LT_{50} values were slightly higher
270 than those estimated for *P. nigra*. *Pinus pinaster* had lower frost survival than *Pinus uncinata*,
271 *P. sylvestris* and *P. nigra*, but higher frost survival than the Mediterranean pines *P. halepensis*
272 and *P. pinea*, which showed little frost survival differences between them.

273

274 *Foliage electrolyte leakage*

275 Across species, a single -15 °C frost increased foliage EL relative to unfrozen plants,
276 particularly in *P. halepensis* and *P. pinea* (Figure 2). However, the effect of the frost on EL
277 depended on seedling ψ_{pd} and the species (Species \times Frost \times ψ_{pd} interaction, Table 1). Thus,
278 among the unfrozen seedlings, reduction in ψ_{pd} increased EL in *P. nigra* and *P. sylvestris*,
279 while EL remained low and was unaffected by ψ_{pd} in the remaining species. Among the
280 frozen plants, species differences in EL depended on seedling water status. Frozen *P.*
281 *halepensis* seedlings had the highest EL values and reduction in ψ_{pd} did not affect EL.
282 Reduction in seedling ψ_{pd} strongly decreased EL after frost in *P. pinea* and to a lesser extent
283 in *P. sylvestris*. In contrast, reduction of seedling ψ_{pd} increased foliage EL after frost in *P.*
284 *uncinata*. Finally, reduction in ψ_{pd} had no effect on EL after frost in *P. pinaster* and *P. nigra*.

285

286 *Percentage loss in stem hydraulic conductivity (PLC) and tracheid diameter*

287 Frost and ψ_{pd} did not affect PLC (Table 1). However, *Pinus uncinata*, *P. nigra* and to a less
288 extent *P. sylvestris*, tended to have slightly higher PLC values than the rest of the species
289 (Table 2).

290 Species differed in tracheid diameter ($P < 0.001$; Table 2). *Pinus uncinata* had the narrowest
291 tracheids followed by *P. halepensis*. In contrast *P. pinaster* and *P. pinea* had the widest
292 tracheids while *P. nigra* and *P. sylvestris* had intermediate tracheid diameter. We did not find
293 any significant correlation between PLC and ψ_{pd} (*P. halepensis*: $r = -0.17$, $p = 0.35$; *P. pinea*:
294 $r = 0.064$, $p = 0.72$; *P. pinaster*: $r = -0.06$, $p = 0.73$; *P. nigra*: $r = 0.18$, $p = 0.33$; *P. sylvestris*: $r = 0.16$,
295 $p = 0.36$; *P. uncinata*: $r = -0.33$, $p = 0.05$).

296

297 *Soluble sugars*

298 Four soluble sugars were detected in the foliage in the following concentration trend: glucose
299 > fructose > galactose > raffinose. A complex of sucrose, maltose and lactose (SML) was also
300 detected in all species. Raffinose occurred at a very low concentration, it was found in all
301 species but *P. pinaster* and no species differences in raffinose were observed (Table 2).
302 Galactose was only found in *P. pinaster* ($7.51 \pm 3.9 \text{ mg g}^{-1}$). The total concentration of soluble
303 sugars, SML and glucose and fructose showed significant differences among species
304 ($P < 0.0001$) *Pinus halepensis* and *P. pinaster* had overall the highest concentration while *P.*
305 *nigra* and *P. sylvestris* had the lowest concentrations. Across species, total concentration of
306 soluble sugar tended to increase with LT_{50} , but the correlation was not statistically significant
307 ($r = 0.59$, $P = 0.22$).

308

309 **Discussion**

310 *Differences in seedling frost survival are related to the natural distribution of pine species in*
311 *Europe.*

312 Juveniles of the pine species studied showed remarkable differences in their ability to survive
313 freezing temperatures. These differences in survival are largely consistent with their range in
314 the Iberian Peninsula, suggesting that the distribution of studied pine species is in part
315 controlled by low temperatures. Our frost survival results agree with the distribution of these
316 species at European scale; but the use of one provenance per species restricted to the Iberian
317 Peninsula requires some caution. *Pinus sylvestris* reaches very high latitudes and together
318 with *P. uncinata* and *P. nigra* constitute the tree line in many mountains in the Iberian
319 Peninsula and in other areas in southern Europe (Barbero et al. 1998; Strimbeck and Schaberg
320 2009). These pine species had LT_{50} values $< -21 \text{ }^\circ\text{C}$. On the contrary, *P. halepensis* and *P.*
321 *pinea* showed almost two-fold higher LT_{50} values than the pines of the cold winter zones; a
322 single $-10 \text{ }^\circ\text{C}$ frost may cause 30% mortality in both species. The low frost tolerance of *P.*

323 *halepensis* and *P. pinea* is consistent with their distribution in mild to cool-winter locations in
324 southern Europe and indicate that strong frosts likely limit the colonization of these
325 Mediterranean pines at higher latitude and altitude locations. Consistent with our findings,
326 Climent et al. (2009) observed a negative relationship between the needle frost damage and
327 the mean temperature of the coldest month of the seed source across several pine species.
328 Similarly, comparing several provenances, Bachofen et al (2016) observed that *P. halepensis*
329 needles were less frost tolerant than *P. sylvestris* and *P. nigra* needles.

330 An unexpected result was that *P. nigra* had a slightly higher frost tolerance than *P.*
331 *sylvestris* and *P. uncinata*. Climent et al (2009) also observed that *P. nigra* secondary needles
332 were less frost damaged than *P. sylvestris* needles. In the high mountains of southern Europe,
333 *P. nigra* is distributed slightly below the *P. sylvestris* and clearly below *P. uncinata* altitudinal
334 belts (Tapias et al. 2011), which would imply that *P. nigra* should be less frost tolerant than
335 *P. sylvestris* and *P. uncinata*. This conflict between frost tolerance and the altitudinal
336 distribution of *P. nigra* may be explained by provenances used in the study or species
337 differences in competitive capacity at the coldest sites and/or the lower capacity of *P.*
338 *sylvestris* and *P. uncinata* to withstand summer drought (Herrero et al. 2013, Matias et al.
339 2017, Tíscar et al. 2017). Drought usually increases at lower altitudes in the mountains of
340 southern Europe (Barbero et al. 1998).

341 Frost tolerance was significantly higher in *Pinus pinaster* than in two species with
342 which it frequently coexist, *P. halepensis* and *P. pinea*. *Pinus pinaster* has remarkable
343 interpopulation differences in frost tolerance with the provenances of cold continental
344 climates having higher frost tolerance than the mild winter sources (Corcuera et al. 2011). In
345 this study, the seeds used to cultivate *P. pinaster* plants come from a cold winter site (Table
346 S1), which may explain the observed high frost tolerance of this species. Similarly, we
347 selected rather cold and continental provenances for *P. halepensis* and *P. pinea* and

348 provenances for *P. sylvestris* and *P. nigra* near the rear edge of their southern distribution in
349 Europe, which might have contributed to reduce species differences in frost tolerance.
350 Consequently, results in this study probably show a conservative measurement of the cold
351 tolerance differences among studied species.

352

353 *Frost tolerance differences among pine species is explained by needle frost*

354 Our results provide insights into the mechanisms underlying the differences in frost survival
355 among pines. Electrolyte leakage is a measure of cell membrane integrity after subjecting
356 plants to stress (Earnshaw, 1993). We found that the two Mediterranean pines, *P. halepensis*
357 and *P. pinea*, which showed the lowest frost survival presented the highest needle electrolyte
358 leakage values after a frost of -15 °C (particularly at high ψ_{pd} , Figure 2). This indicates that the
359 low frost survival of the Mediterranean pines is mostly due to differences in the vulnerability
360 of foliage to low temperature. We cannot exclude that differences in other organs, such as
361 roots also contribute to the observed species differences in frost survival. Toca et al (2017)
362 reported that root frost tolerance in *P. halepensis* and *P. pinea* is lower than in *P. pinaster* and
363 much lower than in *P. nigra*.

364 Glucose and fructose were the most important sugars in the SS pool in all species,
365 consistent with previous findings in other conifers (Hoch et al. 2003). In contrast, raffinose
366 concentration remained low in all species, which contrasts with results in Strimbeck and
367 Schaberg (2009), who reported high leaf concentrations of raffinose and that seasonal
368 increase in raffinose was positively associated to higher frost tolerance in several conifer
369 species. Similar to our findings, Hoch et al. (2003) observed very low levels of raffinose at
370 the end of the winter in *P. sylvestris*. The concentration of SS in plant tissues increases during
371 cold acclimation of perennial plants in temperate and cold biomes to a maximum in the
372 middle of the winter, coinciding with the period of greatest frost tolerance (Chomba et al.

1993, Cavender-Bares et al. 2005, Tjoelker et al. 2008). SS increase the fluidity and stability of the cellular membranes, which prevents tissue freezing damage (Sakai and Larcher 1987, Uemura and Steponkus 2003). Contrary to our expectation we did not find SS concentration to increase with frost tolerance across pine species. Rather, pines that live in mild or cool winter sites tended to have higher SS concentration than cold winter pines (Table 2). Most of the studies that have demonstrated a relationship between frost tolerance and SS are at the intraspecific scale (Hinesley et al. 1992, Ögren et al. 1997, Charrier et al. 2013). For example, Charrier et al. (2013) observed SS to increase with frost tolerance in 9 out of 11 tree species. In contrast to intraspecific comparisons, interspecific studies on the relation between frost tolerance and tissue SS are relatively scarce. Among *Quercus* species no clear relationship was observed between frost tolerance and tissue SS (Cavender-Bares et al. 2005, Morin et al. 2007). Overall, these results suggest that SS do not necessarily explain interspecific differences in frost tolerance. It is possible that compounds other than SS, such as specific proteins (Kontunen-Soppela et al. 2000), might be more important to explain frost tolerance across species.

Plants from cold climates tend to have higher respiration rates than their counterparts from mild climates (Mariko and Koizumi 1993, Reich et al. 1996, Heskell et al. 2016), which may lead to higher non-structural carbohydrate consumption under cool conditions. As the plants used in our study were cultivated in an open nursery, where the mean air temperature varied between 0 and 8 °C most of the winter, species differences in respiration and probably in photosynthesis may explain the lower SS concentration attained by the most frost tolerant species (*P. uncinata*, *P. sylvestris* and *P. nigra*) relative to frost intolerant ones (*P. halepensis* and *P. pinea*). Similarly, growth differences among species were large (Table S2), which may have affected the species SS differences.

397

398 In contrast to needle frost sensitivity differences, the very low PLC values and the lack
399 of species differences in PLC after exposure to -15°C indicate that freezing induced xylem-
400 embolism does not explain frost survival differences among pine species. Vulnerability to
401 freezing-induced embolism is directly related to the diameter of xylem conduits and increases
402 with decreasing xylem water potential (Davis et al. 1999, Pittermann and Sperry 2003, Sperry
403 1995). Xylem conduits in conifers are much narrower and consequently safer than
404 angiosperm vessels (Pittermann and Sperry 2006; Cavender-Bares 2005). We observed that
405 species differences in tracheid diameter were relative small, with average values ranging from
406 6.6 to 10.6 μm and unrelated to native PLC (Table 2). For frost-induced embolisms to occur
407 in such narrow tracheids, xylem water potential must be $< -2.0\text{ MPa}$ (Pittermann and Sperry
408 2006), which is significantly lower than both the lowest ψ_{pd} to which the plants were subjected
409 in our study and the ψ_{pd} that these species commonly experience in the field during winter
410 (Martinez-Ferri et al. 2004, Poyatos et al. 2008, Peguero-Pina et al. 2011).

411 A very interesting result of our study was that a moderate increase in drought stress
412 (ψ_{pd} up to -1.5 MPa) modulated the effect of frost on foliage damage. However, this effect
413 differed among species (Figure 2) and, apparently, it was not associated with species ecology.
414 Drought stress significantly reduced frost damage in *P. sylvestris* and, especially, in *P. pinea*.
415 However, it slightly increased it in *P. uncinata* and had minor effects in the rest of the
416 species. A similar reduction in frost damage in drought stressed *P. sylvestris* plants was
417 reported by Sutinen et al. (1992). However, our results for *P. sylvestris* are not consistent with
418 the interpretation by Peguero-Pina et al. (2011). They mentioned that defoliation in *P.*
419 *silvestris* population at the southern edge of the species range during a dry winter, could be
420 because drought-stressed trees were more predisposed to frost damage than relatively
421 hydrated trees.

422 It is important to note that the EL values after freezing to -15 °C in *P. pinea* seedlings
423 subjected to $\psi_{pd} < -1$ MPa were similar to the EL values of the most frost tolerant pine species
424 at high ψ_{pd} . Similarly, Villar-Salvador et al. (2013) observed that *P. pinea* seedlings subjected
425 to moderate drought stress cycles showed higher tolerance to frost than well-watered plants.
426 The increase in frost tolerance when plants are subjected to moderate drought stress may
427 explain that *P. pinea* can develop in sandy soils in the north of the Iberian Peninsula where
428 the winters are colder than those experienced by *P. pinea* throughout much its range
429 (Campelo et al. 2006). Increases in frost tolerance with drought can be due to activation of
430 generic stress tolerance physiological mechanisms such as abscisic acid or changes in cell
431 membrane composition in lipids and proteins induced by either frost or drought stress
432 (Mäntylä et al. 1995, Pearce 2001, Shinozaki et al. 2003).

433

434 **Conclusions**

435 Our study evidences remarkable differences in the ability of juveniles of six common pines in
436 Europe to survive to frosts, which were related to the winter climatic conditions within their
437 Iberian range. This supports the idea that frost is an important filter for the distribution of the
438 studied pine species in the Iberian Peninsula and likely in the rest of Europe. The
439 Mediterranean pines, *P. halepensis* and *P. pinea* were the least frost tolerant species, while *P.*
440 *sylvestris*, *P. uncinata* and *P. nigra*, which inhabit the coldest locations, were the most frost
441 tolerant species. Interspecific differences in frost tolerance were due to differences in the frost
442 tolerance of foliage, and not to different vulnerability to freezing-induced xylem embolism.
443 Drought increased frost tolerance in *P. pinea* and *P. sylvestris*, but decreased it in *P. uncinata*.
444 Species showing high frost survival had similar or lower concentration of soluble sugars than
445 species having a low frost tolerance, indicating that other elements are more important than
446 soluble sugars in determining differences in frost tolerance across pine species. Our results

447 suggest that the expected increase in winter temperatures associated to climate change will
448 reduce the cold limitations of Mediterranean pines to colonize sites at higher latitude and
449 altitude in Europe.

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462

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723 Table 1. Model results of the effect of predawn water potential, frost, species and their
 724 interactions on the needle electrolyte leakage (EL) and percentage loss in stem hydraulic
 725 conductivity (PLC) in six pine species.

Effect	Needle EL			PLC		
	DF	χ^2	<i>p</i> -value	DF	F value	<i>p</i> -value
Predawn water potential (ψ_{pd})	1	5.43	0.020	1	0.0119	0.91
Frost	1	168	< 0.0001	1	0.881	0.35
Species	5	39.4	< 0.0001	5	2.26	0.050
$\psi_{pd} \times$ Frost	1	4.45	0.035	1	1.66	0.20
Frost \times Species	5	56.0	< 0.0001	5	0.615	0.69
$\psi_{pd} \times$ Species	5	10.7	0.060	5	1.35	0.24
$\psi_{pd} \times$ Frost \times Species	5	25.2	< 0.0001	5	0.693	0.63

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738 Table 2. Percent loss in stem hydraulic conductivity (PLC) after a single -15 °C treatment, and
 739 tracheid diameters and concentration of different foliage soluble sugars of unfrozen, well-
 740 watered plants of six Iberian pine species. Data are mean \pm SE. Same letter indicates not
 741 significant differences. SML: sucrose+maltose+lactose.
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Specie	PLC (%)	Tracheid diameter (μ m)	Soluble sugars (mg g ⁻¹)					Total
			Raffinose	Glucose	Fructose	Fructose	SML	
<i>P. halepensis</i>	15.0 \pm 1.24 c	7.6 \pm 0.44 bc	0.53 \pm 0.42	41.0 \pm 5.9 a	30.2 \pm 6.3 a	1.8 \pm 0.4 a	1.8 \pm 0.4 a	73.5 \pm 11.3 ab
<i>P. pinea</i>	16.6 \pm 1.28 bc	10.5 \pm 0.3 a	0.31 \pm 0.49	34.7 \pm 5.2 ab	27.9 \pm 3.9 ab	0.9 \pm 0.3 b	0.9 \pm 0.3 b	63.8 \pm 8.4 abc
<i>P. pinaster</i>	16.0 \pm 0.89 bc	10.6 \pm 0.63 a	Not detected	41.0 \pm 11 a	30.6 \pm 4.2 a	0.9 \pm 0.3 b	0.9 \pm 0.3 b	80.0 \pm 12.3 a
<i>P. nigra</i>	21.8 \pm 1.35 a	9.5 \pm 0.72 ab	0.61 \pm 0.66	26.3 \pm 8.9 ab	23.1 \pm 6.1 ab	0.2 \pm 0.3 c	0.2 \pm 0.3 c	50.2 \pm 14 c
<i>P. sylvestris</i>	20.0 \pm 1.22 ab	9.6 \pm 0.32 ab	0.80 \pm 0.57	22.4 \pm 8.0 b	19.9 \pm 4.2 b	0.1 \pm 0.2 c	0.1 \pm 0.2 c	43.2 \pm 10.5 c
<i>P. uncinata</i>	23.8 \pm 1.24 a	6.6 \pm 0.37 c	0.33 \pm 0.47	31.5 \pm 8.5 ab	24.7 \pm 5.2 ab	0.1 \pm 0.2 c	0.1 \pm 0.2 c	56.6 \pm 11.7 bc

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750 **Figure legends**

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752 Fig. 1. Relation between survival and freezing temperatures among six pine species
753 distributed in Europe. Data inserted in figure are mean temperature (°C) values and the 95%
754 confidence interval (in brackets). See Material and Methods for data fitting details.

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756 Figure 2. Variation of foliage electrolyte leakage with seedling ψ_{pd} in unfrozen (upper panel)
757 and frozen seedlings (subjected to a single -15 °C frost, lower panel) in six pine species. Data
758 are least square means \pm SE, estimated at -0.4, -1.0 and -1.5 MPa after ANCOVA.

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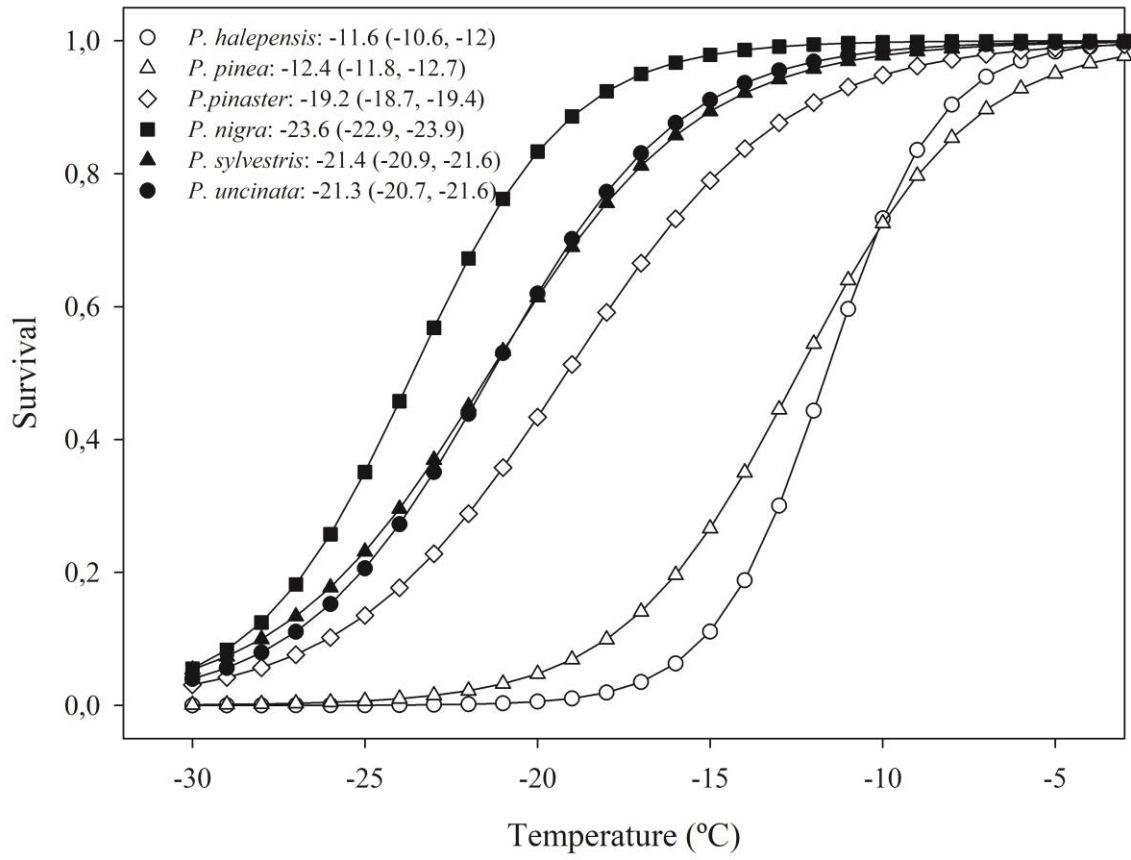
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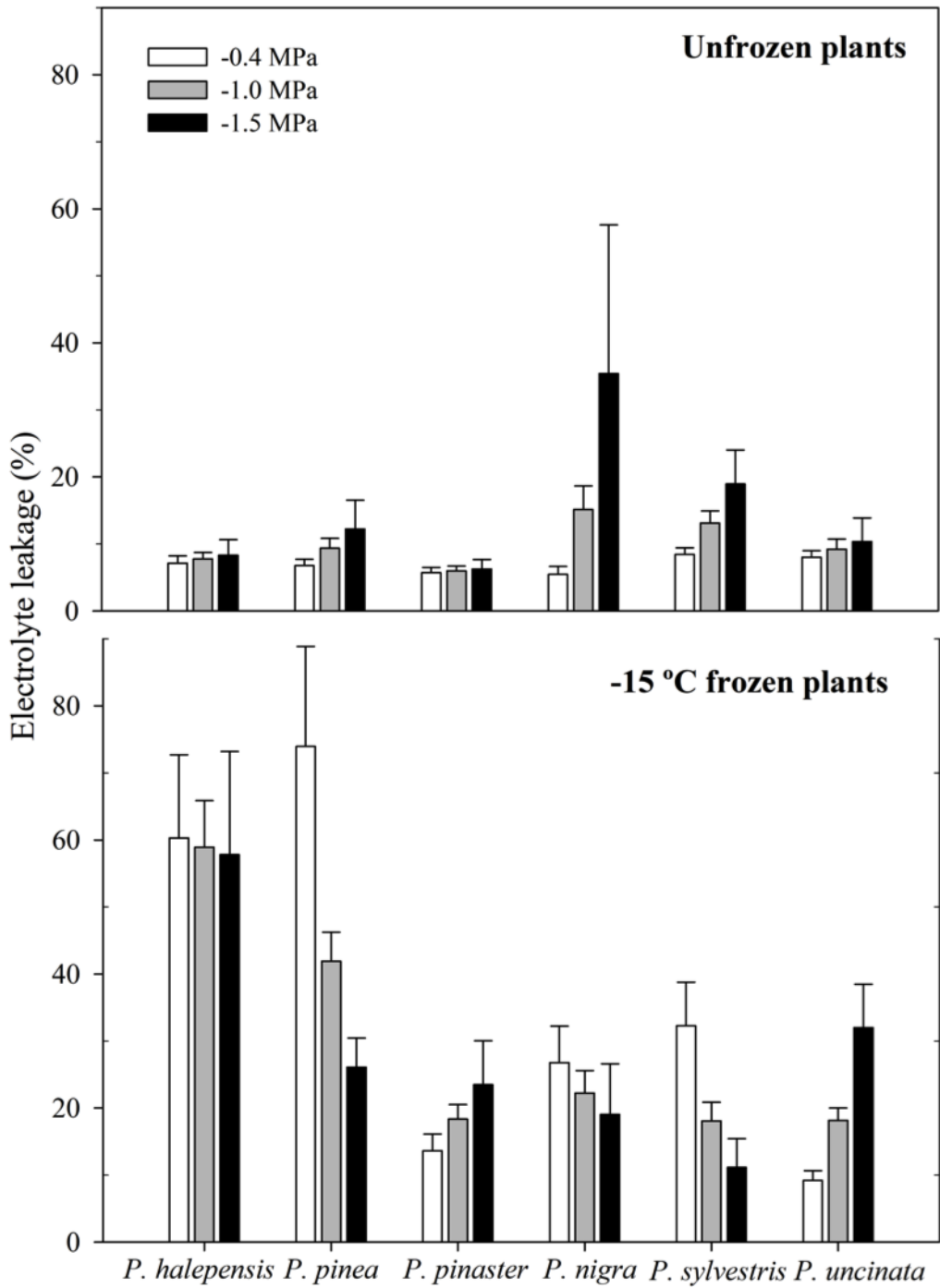
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788 **Supplementary material**

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791 Figure S1. Range and altitudinal segregation (inserted pictograph) of the natural stands of
792 pine species native to Spain. Yellow symbols represent the species in the pictograph and the
793 location of the seed sources of each species used to cultivate the seedlings. Source: Third
794 National Forestry Inventory of the Spanish government
795 [http://www.mapama.gob.es/es/biodiversidad/servicios/banco-datos-naturaleza/informacion-](http://www.mapama.gob.es/es/biodiversidad/servicios/banco-datos-naturaleza/informacion-disponible/ifn3.aspx)
796 [disponible/ifn3.aspx](http://www.mapama.gob.es/es/biodiversidad/servicios/banco-datos-naturaleza/informacion-disponible/ifn3.aspx)

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800 Table S1. Geographic location and climatic characteristics of the provenances where seeds of
801 the study were collected. MAT= mean annual temperature, TCM= mean of the minimum
802 temperatures of the coldest month. Provenance names follow nomenclature in Alía et al
803 (2009)*.

Species	Provenance	Latitude (N)	Longitude (W)	Altitude (masl)	MAT (°C)	TCM (°C)	Annual rainfall (mm)
<i>P. halepensis</i>	Alcarria	40°24'52''	2°24'33''	860	12.6	-0.6	580
<i>P. pinea</i>	La Mancha	39°12'02''	1°57'59''	675	14.2	0.7	397
<i>P. pinaster</i>	Cuenca	39°38'44''	1°13'52''	1135	12	-1.5	540
<i>P. nigra</i> subsp. <i>salzmanii</i>	Sistema Ibérico Meridional	40°15'16''	1°58'22''	1515	10.4	-3.2	617
<i>P. sylvestris</i>	Montes Universales	40°28'09''	1°38'42''	1725	9.2	-4.8	894
<i>P. uncinata</i>	Pirineo Central	42°25'59''	1°40'18''	2050	5.3	-7.5	888

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Table S2. Size of the shoot fractions of the saplings of the six Iberian pine species used in the study. Data are mean \pm SE. Same letter indicates not significant differences (Posthoc test HSD Tukey).

Species	Stem (g)	Needles (g)	Total needles (area cm ²)	Stem diameter (mm)
<i>P. halepensis</i>	2.33 \pm 0.64 b	3.70 \pm 0.84 bc	308 \pm 66 ab	3.6 \pm 0.4 b
<i>P. pinea</i>	5.06 \pm 1.00 a	5.65 \pm 0.84 a	341 \pm 49 a	4.8 \pm 0.6 a
<i>P. pinaster</i>	1.86 \pm 0.38 bc	4.58 \pm 0.5 ab	259 \pm 19 b	3.8 \pm 0.4 b
<i>P. nigra</i>	1.69 \pm 0.45 bc	2.96 \pm 0.63 cd	182 \pm 31 c	3.6 \pm 0.3 b
<i>P. sylvestris</i>	1.84 \pm 0.35 bc	2.24 \pm 0.48 de	138 \pm 20 cd	3.6 \pm 0.2 b
<i>P. uncinata</i>	0.86 \pm 0.37 c	1.65 \pm 0.27 e	94 \pm 28 d	3.2 \pm 0.5 b

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